

# **APPENDIX B**

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**APPLICATION FOR LETTERS PATENT**

for

**PRODUCTION OF VACCINES**

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Title: ~~Production of vaccines~~ TITLE OF THE INVENTION

## PRODUCTION OF VACCINES

### ~~FIELD OF THE INVENTION~~

[0001] Field of the Invention: The invention relates to the development and manufacturing of vaccines. In particular the invention relates to the field of production of viral proteins and/or viruses, more in particular to the use of a mammalian cell, preferably a human cell for the production of viruses growing in eukaryotic, preferably mammalian and, in particular, human cells. The invention is particularly useful for the production of vaccines to aid in protection against viral pathogens for vertebrates, in particular mammals and especially humans.

[0002] Means and methods are disclosed herein for producing a virus and/or viral protein in a (human) cell, preferably using a defined synthetic medium, and for purifying the virus and/or components thereof from the cell and/or culture medium. Pharmaceutical compositions containing virus or its components and methods for manufacturing and recovering and/or purifying them are provided.

### ~~BACKGROUND~~

[0003] Background: Vaccination is the most important route of dealing with viral infections. Although a number of antiviral agents ~~is~~are available, typically these agents have limited efficacy. Administering antibodies against a virus may be a good way of dealing with viral infections once an individual is infected (passive ~~immunisation~~immunization) and typically human or ~~humanised~~humanized antibodies do seem promising for dealing with a number of viral infections, ~~but~~But the most efficacious and safe way of dealing with virus infection is, and probably will be, prophylaxis through active ~~immunisations~~immunizations. Active ~~immunisation~~immunization is generally referred to as vaccination and vaccines ~~comprise~~comprising at least one antigenic determinant of a virus, preferably a number of different antigenic determinants of at least one virus, ~~e.g.e.g.~~e.g., by incorporating in the vaccine at least one viral polypeptide or protein derived from a virus (subunit ~~vaccins~~vaccines). Typically, the formats mentioned ~~so far~~so far include adjuvants in order to enhance an immune response. This also is possible for vaccines based on whole virus, ~~e.g.e.g.~~e.g., in an inactivated format. A further possibility is the use of live, but attenuated forms of the pathogenic virus. A further

possibility is the use of wild-type virus, ~~e.g.e.g.~~, in cases where adult individuals are not in danger from infection, but infants are and may be protected through maternal antibodies and the like. Production of vaccines is not always an easy procedure. In some cases the production of viral material is on eggs, which leads to ~~difficult to purify~~difficulty in purifying material and extensive safety measures against contamination, etc. Also production on bacteria and or yeasts, which sometimes, but not always, is an alternative for eggs, requires many purification and safety steps. Production on mammalian cells would be an alternative, but mammalian cells used so far all require, for instance, the presence of serum and/or adherence to a solid support for growth. In the first case, again, purification and safety and ~~e.g.e.g.~~, the requirement of protease to support the replication of some viruses ~~become~~becomes an issue. In the second case, high yields and ease of production become a further issue. The present invention overcomes at least a number of the problems encountered with the production systems for production of viruses and/or viral proteins for vaccine purposes of the systems of the prior art.

#### DESCRIPTION-BRIEF SUMMARY OF THE INVENTION

[0004] Thus, the invention provides a method for producing a virus and/or viral proteins, other than adenovirus or adenoviral proteins, for use as a vaccine comprising providing a cell with at least a sequence encoding at least one gene product of the E1 gene or a functional derivative thereof of an adenovirus, providing ~~said the~~ cell with a nucleic acid encoding ~~said the~~ virus or ~~said the~~ viral proteins, culturing ~~said the~~ cell in a suitable medium and allowing for propagation of ~~said the~~ virus or expression of ~~said the~~ viral proteins and harvesting ~~said the~~ virus and/or viral proteins from ~~said the~~ medium and/or ~~said the~~ cell. Until the present invention there are few, if any (human) cells that have been found suitable to produce viruses and/or viral proteins for use as vaccines in any reproducible and upscaleable manner and/or with sufficiently high yields and/or which are easily purifiable. We have now found that cells which comprise adenoviral E1 sequences, preferably in their genome, are capable of sustaining the propagation of viruses in significant amounts.

[0005] The preferred cell according to the invention is derived from a human primary cell, preferably a cell which is ~~immortalised~~immortalized by a gene product of ~~said the~~ E1 gene. In order to be able to grow a primary cell, of course, it needs to be immortalized. A good example of such a cell is one derived from a human embryonic retinoblast.

[0006] In cells according to the invention, it is important that the E1 gene sequences are not lost during the cell cycle. It is, therefore, preferred that ~~said-the~~ sequence encoding at least one gene product of the E1 gene is present in the genome of ~~said-the~~ (human) cell. For reasons of safety care, it is best taken to avoid unnecessary adenoviral sequences in the cells according to the invention. It is, thus, another embodiment of the invention to provide cells that do not produce adenoviral structural proteins. However, in order to achieve large scale (continuous) virus production through cell culture, it is preferred to have cells capable of growing without needing anchorage. The cells of the present invention have that capability. To have a clean and safe production system from which it is easy to recover and, if desirable, to purify the virus, it is preferred to have a method according to the invention, whereby ~~said-the~~ human cell comprises no other adenoviral sequences. The most preferred cell for the methods and uses of the invention is PER.C6, as deposited under ECACC no. 96022940, or a derivative thereof.

[0007] Thus, the invention provides a method of using a cell according to the invention, wherein ~~said-the~~ cell further comprises a sequence encoding E2A, or a functional derivative or analogue or fragment thereof, preferably, a cell wherein ~~said-the~~ sequence encoding E2A, or a functional derivative or analogue or fragment thereof is present in the genome of ~~said-the~~ human cell and, most preferably, a cell wherein ~~said-the~~ E2A encoding sequence encodes a ~~temperature-temperature-sensitive~~ mutant E2A.

[0008] Furthermore, as stated, the invention also provides a method according to the invention wherein ~~said-the~~ (human) cell is capable of growing in suspension.

[0009] The invention also provides a method wherein ~~said-the~~ human cell can be cultured in the absence of serum. The cells according to the invention, in particular PER.C6, have ~~has~~ the additional advantage that ~~it~~they can be cultured in the absence of serum or serum components. Thus, isolation is easy, safety is enhanced and reliability of the system is good (synthetic media are the best in reproducibility). The human cells of the invention and, in particular, those based on primary cells and particularly the ones based on HER cells, are capable of normal post- and ~~peri~~peri-translational modifications and assembly. This means that they are very suitable for preparing viral proteins and viruses for use in vaccines.

[0010] Thus, the invention provides a method according to the invention, wherein ~~said-the~~ virus and/or ~~said-the~~ viral proteins comprise a protein that undergoes post-translational and/or ~~peri~~peri-translational modification, especially wherein ~~said-the~~ modifications comprise

glycosylation. A good example of a viral vaccine that has been cumbersome to produce in any reliable manner is influenza vaccine. The invention provides a method ~~according to the invention~~ wherein ~~said the~~ viral proteins comprise at least one of an Influenza virus neuramidase and/or a ~~haemagglutinin~~hemagglutinin. Other viral proteins (subunits) and viruses (wild-type to be inactivated) or attenuated viruses that can be produced in the methods according to the invention include enterovirus, such as rhinovirus, aphthovirus, or ~~poliomyelitis virus~~poliomyelitis virus, ~~herpes virus~~herpes virus, such as herpes simplex virus, pseudorabies virus or bovine herpes virus, orthomyxovirus, such as influenza virus, a paramyxovirus, such as ~~newcastle~~Newcastle disease virus, respiratory syncytio virus, mumps virus or a measles virus, retrovirus, such as human immunodeficiency virus, or a parvovirus or a papovavirus, rotavirus or a coronavirus, such as transmissible ~~gastroenteritis virus~~gastroenteritis virus or a flavivirus, such as tick-borne encephalitis virus or yellow fever virus, a togavirus, such as rubella virus or eastern-, western-, or ~~venezuelan~~Venezuelan-equine encephalomyelitis virus, a hepatitis causing virus, such as hepatitis A or hepatitis B virus, a pestivirus, such as hog cholera virus, or a rhabdovirus, such as rabies virus.

[0011] The invention also provides the use of a human cell having a sequence encoding at least one E1 protein of an adenovirus or a functional derivative, homologue or fragment thereof, in its genome, which cell does not produce structural adenoviral proteins for the production of a virus, or at least one viral protein for use in a vaccine. Of course, for such a use the cells preferred in the methods according to the invention are also preferred. The invention also provides the products resulting from the methods and uses according to the invention, especially viral proteins and viruses obtainable according to those uses and/or methods, especially when brought in a pharmaceutical composition comprising suitable excipients and in some formats (inactivated viruses, subunits) adjuvants. Dosage and ways of administration can be sorted out through normal clinical testing in as far as they are not yet available through the already registered vaccines.

[0012] Thus, the invention also provides a virus or a viral protein for use in a vaccine obtainable by a method or by a use according to the invention, ~~said the~~ virus or ~~said the~~ viral protein being free of any non-human mammalian proteinaceous material, and a pharmaceutical formulation comprising such a virus and/or viral protein.

[0013] The invention further provides a human cell having a sequence encoding at least one E1 protein of an adenovirus or a functional derivative, homologue or fragment thereof, in its genome, which cell does not produce structural adenoviral proteins and having a nucleic acid encoding a virus or at least one non-adenoviral viral protein. This cell can be used in a method according to the invention.

[0014] In a preferred embodiment, the invention provides influenza virus obtainable by a method according to the invention or by a use according to the invention. In another embodiment the invention provides influenza vaccines obtainable by a method according to the invention or by a use according to the invention.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0048] Figure 1: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay versus percentage of dead cells measured via FACS after propidium iodide staining, at moi of 10<sup>-3</sup> and 10<sup>-4</sup>. Poor viability of the cells from samples derived from infection at moi 10<sup>-3</sup> didn't give rise to reliable data.

[0049] Figure 2: Percentage of infected cells viewed microscopically after immunofluorescence assay. Samples derived from infection at moi 10 and 1, at 48h post infection are not shown, because of full CPE.

[0050] Figure 3: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 to day 6 after infection.

[0051] Figure 4: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.

[0052] Figure 5: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 to 6 after infection.

[0053] Figure 6: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.

[0054] Figure 7: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 2 to day 6 after infection.

## ~~Detailed description.~~ DETAILED DESCRIPTION OF THE INVENTION

[0015] The present invention discloses a novel<sub>1</sub> human immortalized cell line for the purpose of propagating and harvesting virus, for production of ~~said the~~ virus. PER.C6 cells (WO 97/00326) were generated by transfection of primary human embryonic retina cells, using a plasmid that contained the Ad serotype 5 (Ad5) E1A- and E1B-coding sequences (Ad5 nucleotides 459-3510 SEQ ID NO:1) under the control of the human phosphoglycerate kinase (PGK) promoter.

[0016] The following features make PER.C6<sub>1</sub> or a derivative<sub>1</sub> particularly useful as a host for virus production: it is a fully characterized human cell line<sub>2</sub>; it was developed in compliance with GLP<sub>2</sub>; it can be grown as suspension cultures in defined serum-free medium, devoid of any human or animal serum proteins; and its growth is compatible with roller bottles, shaker flasks, spinner flasks and bioreactors, with doubling times of about 35 hrs<sub>2</sub>.

### Influenza epidemiology:

[0017] Influenza viruses, members of the family of ~~Orthomyxoviridae~~ Orthomyxoviridae, are the causative agents of annual epidemics of acute respiratory disease. In the US alone, 50 million Americans get the flu each year. Estimated deaths worldwide (1972-1992) are ~~60.000~~ 60,000 (CDC statistics). There have been 3 major cases of ~~pandemics~~ pandemic outbreaks of influenza, namely in 1918 (Spanish flu, ~~est.estimated~~ 40 million deaths), in 1957 (Asian flu, ~~est.estimated~~ 1 million deaths), and in 1968 (Hong-Kong flu, ~~est. 700.00~~ estimated 700,000 deaths). Infections with influenza viruses are associated with a broad spectrum of illnesses and complications that result in substantial worldwide morbidity and mortality, especially in older people and patients with chronic illness. Vaccination against influenza is most effective in preventing the often fatal complications associated with this infection (Murphy, B.R and ~~R.G.~~ Webster, R.G., 1996). The production of influenza virus on the diploid human cell line MRC-5 has been reported (Herrero-Euribe L<sub>2</sub> et al<sub>2</sub>, 1983). However, the titers of influenza virus are prohibitively low.

### Strains of Influenza virus

[0018] Present day flu vaccines contain purified ~~Hemagglutinin~~ hemagglutinin and neuraminidase of influenza virus A and B. The 3 viruses that represent epidemiologically



important strains are influenza A(H1N1), influenza A(H3N2) and influenza B. The division into A and B types is based on antigenic differences between their nucleoprotein (NP) and matrix (M) protein antigen. The influenza A virus is further subdivided into subtypes based on the antigenic composition (sequence) of hemagglutinin (H1-H15) and neuraminidase (N1-N9) molecules. Representatives of each of these subtypes have been isolated from aquatic birds, which ~~probable~~probably are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission has been shown between pigs and humans and, recently, (H5N1) between birds and humans.

#### Influenza vaccines

[0019] Three types of inactivated influenza ~~vaccine~~vaccines are currently used in the world: whole virus, split product and surface antigen or subunit vaccines. These vaccines all contain the surface glycoproteins, ~~haemagglutinin~~hemagglutinin (HA) and neuraminidase (NA), of the influenza virus strains that are expected to circulate in the human population in the upcoming season.

[0020] These strains, which are incorporated in the vaccine, are grown in embryonated hens' eggs, and the viral particles are subsequently purified before further processing.

[0021] The need for the yearly adjustment of influenza vaccines is due to antigen variation caused by processes known as “antigenic drift” and “antigenic shift”.

[0022] Antigenic drift occurs by the accumulation of a series of point mutations in either the H or N protein of a virus resulting in amino acid substitutions. These substitutions prevent the binding of neutralizing antibodies, induced by previous infection, and the new variant can infect the host.

[0023] Antigenic shift is the appearance of a new subtype by genetic reassortment between animal and human influenza A viruses. The pandemic strains of 1957 (H2N2) and 1968 (H3N2) are examples of reassorted viruses by which avian H ~~and~~ and/or N genes were introduced in circulating human viruses, which subsequently could spread among the human population.

[0024] Based on the epidemiological surveys by over hundred National Influenza Centers worldwide, the World Health Organization (WHO) yearly recommends the composition of the influenza vaccine, usually in February for the Northern hemisphere, and in September for

the Southern hemisphere. This practice limits the time window for production and standardization of the vaccine to a maximum of 9 months.

[0025] In case of an urgent demand of many doses of vaccine, for example, when a novel subtype of influenza A virus arises by antigenic shift and antigenic drift, limited availability of eggs may hamper the rapid production of vaccine. Further disadvantages of this production system are the lack of flexibility, the risk of the presence of toxins and the risks of adventitious viruses, particularly retroviruses, and concerns about sterility. This presents a serious problem in today's practice of influenza vaccine production on embryonated hens' eggs.

[0026] ~~Therefor~~Therefore, the use of a cell culture system for influenza vaccine production would be an attractive alternative. Influenza viruses can be grown on a number of primary cells, including monkey kidney, calf kidney, hamster kidney and chicken kidney. Yet, their use for vaccine production is not practical, because of the need to re-establish cultures from these primary cells for each preparation of a vaccine. ~~Therefor~~Therefore, the use of continuous cell lines for influenza vaccine production is an attractive alternative.

[0027] The use of culture systems was facilitated by the realization that the proteolytic cleavage of HA in its two subunits (HA1 and HA2), which is required for influenza virus infectivity, can be obtained by the addition of trypsin. Inclusion of trypsin permits replication and plaque formation in Madin-Darby canine kidney (MDCK) cells (Tobita, K., et al., 1975).

[0028] The MDCK cell line was recently shown to support the growth of influenza virus for vaccine production (Brand, R., et al., 1996, 1997; Palache, A.M., et al., 1997). The use of trypsin requires growth of the MDCK cells in serum-free tissue culture medium (MDCK-SF1). However, MDCK cells are currently not approved as a substrate for production of influenza virus.

[0029] However, any non-human system for production of influenza vaccines has an inherent drawback, known as "adaptation". Human influenza A and B virus both carry mutations in the HA, due to adaptation in embryonated hens' eggs. These mutations result in altered antigenicity (Newman, R.W., et al., 1993; Williams, S.P. and Robertson, J.S., 1993; Robertson, J.S., et al., 1994; Gubareva, L.V., et al., 1994; Schild, G.C., et al., 1993; Robertson, J.S., et al., 1987; Kodihalli, S., et al., 1995). In humans, immunization with vaccines containing an HA bearing an egg-adaption mutation induces less neutralizing antibody to virus that contains a non-egg adapted HA (Newman, R.W., et al., 1993).

[0030] Human influenza viruses propagated in canine cells such as MDCK cells also show adaptation, albeit to a lesser extent. Such viruses resemble the original human isolates more closely than egg derived viruses (Robertson, J.S., et al., 1990-~~et al~~).

[0031] Furthermore, there is evidence that host-specific changes in NA and host-specific phosphorylation patterns of NP can affect the replication of influenza viruses (Schulman, J.L. and Palese, P., 1977; Sugiara, A., and Ueda, M., 1980; Kistner, O., et al., 1976).

[0032] ~~Therefor~~Therefore, it would clearly be advantageous to avoid adaptation or other host-induced changes of influenza virus. It may result in a more homogeneous population of viruses and render the ultimate vaccine more effective.

[0033] It is, ~~therefor~~therefore, an object of the present invention to provide human cells as a substrate for the production of high titers of influenza virus, suitable for the development of vaccines.

## EXAMPLES

[0034] To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention.

### PER.C6 Cell banking

[0035] Cell line PER.C6 (deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research), or derivatives thereof, were used (described in WO 97/00326). Cell lines were banked by a two tier cell bank system. The selected cell line was banked in a research master cell bank (rMCB) which was stored in different locations. From this rMCB, working cell banks were prepared as follows: an ~~ampoule~~ampule of the rMCB was thawed, and the cells were propagated until enough cells ~~are~~were present to freeze the cells by using dry ice. 400-500 ~~ampoules~~ampules containing 1 ml (1-2x10<sup>6</sup>cells/ml) of rWCB were stored in the ~~vapour~~vapor phase of a liquid nitrogen freezer.

### PER.C6 preculture

[0036] One ~~ampoule~~ampule containing 5x10<sup>6</sup> PER.C6 cells of the WCB was thawed in a water bath at ~~37°C~~37°C. Cells were rapidly transferred into a ~~50ml~~50 ml tube and resuspended by adding ~~9ml~~9 ml of the suspension medium ~~Ex-Cell™~~ExCell™ 525 (JRH Biosciences,

Denver, Pennsylvania) supplemented with 1 x L-Glutamin. After 3 minutes of centrifugation at 1000 rpm, cells were resuspended in a final concentration of  $3 \times 10^5$  cells/ml and cultured in a ~~T80cm<sup>2</sup>~~T80 cm<sup>2</sup> tissue culture flask, at ~~37°C~~37°C., 10% ~~CO<sub>2</sub>~~CO<sub>2</sub>. ~~Two-three~~Two to three days later, cells were seeded into ~~490cm<sup>2</sup>~~490 cm<sup>2</sup> tissue culture roller bottles (Corning Costar Corporation, Cambridge, USA), with a density of  $3 \times 10^5$ /ml and cultured in continuous rotation at 1 ~~RPM~~rpm.

#### PER.C6 and MDCK Cell culture

[0037] Madin Darby Canine Kidney (MDCK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Breda, The Netherlands) containing 10% heat inactivated fetal bovine serum and 1x L-Glutamin (Gibco-BRL), at ~~37°C~~37°C. and 10% ~~CO<sub>2</sub>~~CO<sub>2</sub>.

[0038] Suspension cultures of PER.C6<sup>TM</sup> were cultured in ~~Ex-Cell<sup>TM</sup>~~ExCell<sup>TM</sup> 525 (JRH Biosciences, Denver, Pennsylvania) supplemented with 1 x L-Glutamin, at ~~37°C~~37°C. and 10% ~~CO<sub>2</sub>~~CO<sub>2</sub>, in stationary cultures in 6 well dishes (Greiner, Alphen aan de Rijn, The Netherlands) or in ~~490cm<sup>2</sup>~~490 cm<sup>2</sup> tissue culture roller bottles (Corning Costar Corporation, Cambridge, USA) during continuous rotation at 1 ~~RPM~~rpm.

#### Immunofluorescence test

[0039] Direct immunofluorescence assays for the detection of influenza virus infection were carried out using the ~~IMAGEN<sup>TM</sup>~~IMAGEN<sup>TM</sup> Influenza Virus A and B kit (DAKO, Glostrup, Denmark) according to the standard protocol of the supplier. Samples were viewed microscopically using epifluorescence illumination. Infected cells are characterized by a bright apple-green fluorescence.

#### Propidium Iodide staining

[0040] Cell pellets were resuspended into 300  $\mu$  of cold ~~PBS-0.5%~~PBS-0.5% BSA + 5  $\mu$  of propidium iodide 50  $\mu$ g/ml in ~~PBS-FCS-azide~~PBS-FCS-azide solution. Viable and dead cells were then detected via flow cytofluorometric analysis.

## Hemagglutination assay

[0041] To 50  $\mu$ l of two fold diluted virus solutions in PBS, 25  $\mu$ l of a 1% suspension of turkey erythrocytes in PBS was added in 96 well microtiter plates and incubated at 4°C for 1h. The hemagglutination pattern was examined, and expressed as hemagglutinating units (HAU). ~~the~~The amount of HAU corresponded to the reciprocal value of the highest virus dilution that showed complete hemagglutination.

## PER.C6 cells as permissive cell line for Influenza A virus

[0042] PER.C6TM is not known for its ability to sustain influenza virus infection and replication. We, therefore, verified whether PER.C6 cells are permissive for influenza virus infection in comparison with MDCK (Madin Darby Canine Kidney) cells.

[0043] The day before infection,  $2 \times 10^5$  MDCK cells/well were seeded in 6-well plates. 24 hours later,  $4 \times 10^5$  PER.C6/well and MDCK were infected with the H1N1 strain A/Puerto Rico/8/34 (titer  $3.6 \times 10^7$  pfu/ml), obtained from Dr. Eric Claas, ~~Dept.~~Department of Virology, Leiden University Medical Center, The Netherlands. Infection was performed at various ~~multiplicities~~multiplicities of infection (~~moi's~~moi) ranging from 0.1 to 10 pfu/cell. After about 2 hours of incubation at ~~37°C~~37°C, the inoculum was removed and replaced by fresh culture medium. A direct immunofluorescence assay for the detection of influenza virus infection was ~~performed~~performed 24 and 48 hours post infection. The experiment showed permissivity of PER.C6 for influenza infection, with percentages of positive cells moi-dependent and comparable with MDCK (see Table 1).

## PER.C6 cells as cell line for Influenza A virus propagation

[0044] We verified whether replication and propagation of influenza virus are supported by PER.C6. The day of infection, PER.C6 cells were seeded in ~~490cm<sup>2</sup>~~2490 cm<sup>2</sup> tissue culture roller bottles, with the density of  $2 \times 10^5$  cells/ml in a final volume of 40ml, in the presence of 5  $\mu$ g/ml of ~~trypsin~~trypsin-EDTA (Gibco-BRL). Cells were either, mock inoculated or infected with the H3N2 strain A/Shenzhen/227/95 (titer  $1.5 \times 10^6$  pfu/ml), a kind gift from Dr. Eric Claas, ~~Dept.~~Department of Virology, Leiden University Medical Center, The Netherlands. Infections were performed at moi 10<sup>-4</sup> and 10<sup>-3</sup> pfu/cell. After 1 hour of incubation at ~~37°C~~37°C, the inoculum was removed by spinning down the cells at 1,500 rpm and resuspending them in fresh

culture medium + 5 ( $\mu\text{g/ml}$  of ~~trypsin~~trypsin-EDTA. Harvest of 1.3 ml of cell suspension was carried out each day, from day 1 to day 6 post-infection. Supernatants were stored at ~~-80°C~~-80°C. and used for hemagglutination assays. Cell pellets were used for direct immunofluorescence tests and for propidium iodide staining- (see Figure 2).

#### Permissivity of PER.C6 for influenza strains

**[0045]** To further investigate the permissivity of PER.C6 for propagation of various influenza strains, we performed an infection by using the H1N1 vaccine strains A/Beijing/262/95 and its reassortant X-127 obtained from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK. The day of infection, PER.C6 cells were seeded in ~~490cm~~<sup>2</sup>490 cm<sup>2</sup> tissue culture roller bottles, with the density of approximately  $1 \times 10^6$  cells/ml in a final volume of 50ml. Cells were inoculated with 5 $\mu\text{l}$  (10<sup>-4</sup> dilution) and 50 $\mu\text{l}$  (10<sup>-3</sup> dilution) of virus in the presence of 5  $\mu\text{g/ml}$  trypsin-EDTA. In order to establish if trypsin was indeed required, one more infection was carried out by inoculating 5 $\mu\text{l}$  of the strain A/Beijing/262/95 in the absence of the protease. After approximately 1 hour of incubation at 37°C, the inoculum was removed by spinning down the cells at 1,500 rpm and resuspending them in fresh culture medium  $\pm$  5  $\mu\text{g/ml}$  of trypsin-EDTA. At day 2 and day 4 post-infection, more trypsin was added to the samples. Harvest of 1.3 ml of cell suspension was carried out from day 1 to day 6 post-infection. Supernatants were stored at -80°C and used for hemagglutination assays and further infections; cell pellets were used for direct immunofluorescence tests. Results obtained with the ~~above~~ above-mentioned immunofluorescence and hemagglutination assays are shown in ~~fig~~Figures 4 and ~~fig~~5, respectively, illustrating the efficient replication and release of the viruses.

#### Infectivity of virus propagated on PER.C6

**[0046]** We verified if the viruses grown in PER.C6 were infectious and if adaptation to the cell line could increase virus yields. Virus supernatants derived from PER.C6 infected with the strains A/Beijing/262/95 and its reassortant X-127 (dil.10<sup>-3</sup>) and harvested at day 6 post-infection, were used. At the day of infection, PER.C6 were seeded in ~~490cm~~<sup>2</sup>490 cm<sup>2</sup> tissue culture roller bottles, with the density of approximately  $1 \times 10^6$  cells/ml in a final volume of 50ml. Cells were inoculated with 100  $\mu\text{l}$  and 1 ml of virus supernatant in the presence of 5  $\mu\text{g/ml}$  trypsin-EDTA. In order to establish if trypsin was still required, one more infection was carried

out by inoculating 100 $\mu$ l of the strain A/Beijing/262/95 in the absence of the protease. After approximately 1 hour of incubation at 37°C, the inoculum was removed by spinning down the cells at 1,500 rpm and resuspending them in fresh culture medium  $\pm$  5  $\mu$ g/ml of trypsin-EDTA. At day 2 and day 4 post-infection, more trypsin was added to the samples. Harvest of 1.3 ml of cell suspension was carried out from day 1 to day 6 post-infection. Supernatants were stored at -80°C and used for hemagglutination assays and further infections; cell pellets were used for direct immunofluorescence tests. Results obtained with the ~~above-mentioned~~above-mentioned immunofluorescence and hemagglutination assays are shown in ~~fig~~Figures 6 and ~~fig~~7, respectively. Data obtained with the present experiment -showed infectivity of the viruses grown in PER.C6 as well as an increase in virus yields.

#### Recovery of virus

[0047] Intact virus is recovered from the culture medium by ion-exchange chromatography. The virus preparations are further processed to an inactivated surface antigen preparation by -formaldehyde ~~inactivation~~inactivation, ~~solubilisation~~solubilization with detergent and ultrafiltration and ultracentrifugation (Bachmayer, H., 1975).

#### LEGENDS TO THE FIGURES

~~[0048]— Fig. 1: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay versus percentage of dead cells measured via FACS after propidium iodide staining, at moi's of 10<sup>-3</sup> and 10<sup>-4</sup>. Poor viability of the cells from samples derived from infection at moi 10<sup>-3</sup> didn't give rise to reliable data.~~

~~[0049]— Fig. 2: Percentage of infected cells viewed microscopically after immunofluorescence assay. Samples derived from infection at moi 10 and 1, at 48h post-infection are not shown, because of full CPE~~

~~[0050]— Fig. 3: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 to day 6 after infection.~~

~~[0051]— Fig. 4: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.~~

~~[0052]— Fig. 5: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 1 to 6 after infection.~~

~~{0053} Fig. 6: Percentage of infected cells (positive cells) viewed microscopically after immunofluorescence assay.~~

~~{0054} Fig. 7: Kinetics of virus propagation measured in hemagglutinating units (HAU) from day 2 to 6 after infection.~~

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## SEQUENCE LISTING

<110> Introgene BV

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<120> Production of Vaccines

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## ABSTRACT

Novel means and methods are provided for the production of mammalian viruses, comprising, infecting a culture of immortalized human cells with the virus, incubating the culture infected with virus to propagate the virus under conditions that permit growth of the virus, and to form a virus-containing medium, and removing the virus-containing medium. \_

The viruses can be harvested and be used for the production of vaccines.\_

Advantages —are that human cells of the present invention can be cultured under defined serum free conditions, and the cells show improved capability for propagating virus.\_

In particular, methods are provided for producing, in cultured human cells, influenza virus and vaccines derived thereof. This method eliminates the necessity to use whole chicken embryos for the production of influenza vaccines.\_

The method provides also for the continuous or batchwise removal of culture media. As such, the present invention allows the ~~large~~-large-scale, continuous production of viruses to a high titer.

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